



A New Quaternary Structure Epitope on Dengue Virus Serotype 2 Is the Target of Durable Type-Specific Neutralizing Antibodies

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ABSTRACT Dengue virus serotype 2 (DENV2) is widespread and responsible for severe epidemics. While primary DENV2 infections stimulate serotype-specific protective responses, a leading vaccine failed to induce a similar protective response. Using human monoclonal antibodies (hMAbs) isolated from dengue cases and structure-guided design of a chimeric DENV, here we describe the major site on the DENV2 envelope (E) protein targeted by neutralizing antibodies. DENV2-specific neutralizing hMAb 2D22 binds to a quaternary structure epitope. We engineered and recovered a recombinant DENV4 that displayed the 2D22 epitope. DENV2 neutralizing antibodies in people exposed to infection or a live vaccine tracked with the 2D22 epitope on the DENV4/2 chimera. The chimera remained sensitive to DENV4 antibodies, indicating that the major neutralizing epitopes on DENV2 and -4 are at different sites. The ability to transplant a complex epitope between DENV serotypes demonstrates a hitherto underappreciated structural flexibility in flaviviruses, which could be harnessed to develop new vaccines and diagnostics.

IMPORTANCE Dengue virus causes fever and dengue hemorrhagic fever. Dengue serotype 2 (DENV2) is widespread and frequently responsible for severe epidemics. Natural DENV2 infections stimulate serotype-specific neutralizing antibodies, but a leading DENV vaccine did not induce a similar protective response. While groups have identified epitopes of single monoclonal antibodies (MAbs), the molecular basis of DENV2 neutralization by polyclonal human immune sera is unknown. Using a recombinant DENV displaying serotype 2 epitopes, here we map the main target of DENV2 polyclonal neutralizing antibodies induced by natural infection and a live DENV2 vaccine candidate. Proper display of the epitope required the assembly of viral envelope proteins into higher-order structures present on intact virions. Despite the complexity of the epitope, it was possible to transplant the epitope between DENV serotypes. Our findings have immediate implications for evaluating dengue vaccines in the pipeline as well as designing next-generation vaccines.

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Dengue virus (DENV) is the most significant arboviral infection of humans, with an estimated 390 million infections and 96 million symptomatic cases annually (1). The DENV complex consists of four distinct serotypes (DENV1 to -4). Infection with one serotype induces long-term protective immunity to the homologous serotype only. In fact, immunity to one serotype is associated with an increased risk of severe disease upon subsequent infection with a different serotype, a confounding factor for vaccine design. Many dengue vaccines in clinical trials are tetravalent live-attenuated virus formulations that are designed to simultaneously induce protective immunity to all 4 serotypes (2–4). However, in phase 3 efficacy trials in Asia and Latin America, the leading vaccine was 50 to 78% efficacious against serotypes 1, 3, and 4

but only 35 to 42% efficacious against serotype 2 (5, 6). Moreover, in vaccinees less than 5 years of age, incidence of hospitalization for virologically confirmed dengue was ~8-fold higher than that seen in matched nonvaccinated controls, demonstrating a critical need for new metrics of protective immunity (7). Here we describe the main site on DENV2 recognized by type-specific and durable neutralizing antibodies in people and other primates exposed to natural infections or a candidate live attenuated DENV2 vaccine.

The DENV envelope (E) glycoprotein is the main target of protective antibodies (8). The E protein is composed of three domains: I, II and III (designated EDI, EDII, and EDIII, respectively). Each DENV particle has 180 monomers of E that are organized into 90 dimers that cover the entire surface of the virus

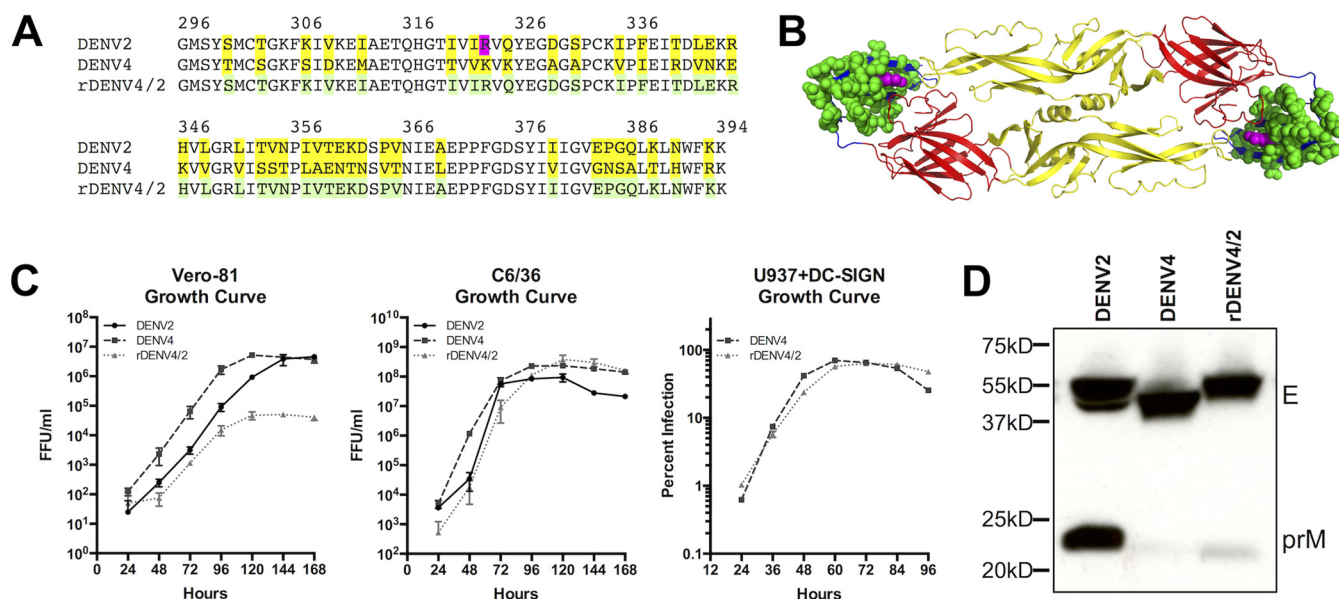


FIG 1 Design and characterization of rDENV4/2. (A) Amino acid alignment of DENV2 and DENV4 linear envelope domain III (EDIII) sequence, showing residues 296 to 395 of the entire E sequence (99 amino acids [aa] total). Residues differing between DENV2 and DENV4 are highlighted in yellow. Recombinant DENV4 virus containing EDIII from DENV2, designated rDENV4/2, replaces differing residues from DENV4 with those from DENV2, highlighted in green (40 aa total). A residue generated from the escape mutant is highlighted in magenta. (B) Crystal structure model of the DENV2 E protein dimer, with swapped residues colored in green and the DENV2 type-specific MAb 2D22 escape mutant residue highlighted in magenta. (C) Vero-81 or C6/36 cells were inoculated, viral supernatants were collected every 24 h, and the viral titer was subsequently determined on the respective cell type, or 1% of U937+DC-SIGN cells were infected, and the total percentage of infection was measured every 12 h (mean \pm SD). (D) Immunoblotting of C3/36-grown viruses with anti-E and anti-PrM antibodies. The molecular mass of E is 55 kDa, and the molecular mass of prM is 21 kDa.

(9). The arrays of E proteins are arranged with icosahedral symmetry, with each asymmetric unit containing three E protein dimers. Some human monoclonal antibodies (hMAbs) that neutralize DENVs bind to quaternary structure epitopes that require assembly of E protein into homodimers or higher-order structures (10–14). Following infection or vaccination, it is a DENV-specific serum polyclonal antibody response that is responsible for protection. The principle targets of the human polyclonal antibody responses that neutralize DENVs have remained elusive.

We recently described hMAb 2D22, which is a DENV2-specific strongly neutralizing antibody isolated from a person exposed to a primary DENV2 infection (10). Our studies also demonstrated that 2D22 recognizes a complex quaternary epitope displayed on the intact virus but not recombinant E protein. A point mutation at amino acid position 323 in EDIII (residue highlighted in magenta in Fig. 1A and B) led to complete escape from 2D22 neutralization, indicating that the epitope includes EDIII residues (10). Recently Fibriansah et al. solved the structure of 2D22 bound to DENV2 and demonstrated that the antibody bound to a quaternary epitope that was formed by EDIII and EDII on two different monomers within a single dimer (15). While MAbs are powerful tools for epitope mapping, it is the polyclonal serum antibody response derived from long-lived plasma cells that is protective in people. Here we demonstrate that 2D22 defines a new class of quaternary epitopes that are the main targets of serum neutralizing antibodies in people exposed to DENV2 infections or a leading live attenuated DENV2 vaccine candidate.

RESULTS

Design of rDENV4/2 chimeric virus. To further understand the role of EDIII in the epitope of 2D22 and DENV2 neutralizing

antibodies in general, we designed and recovered a recombinant chimeric virus in which the entire DENV2 EDIII region was inserted into the backbone sequence of a DENV4 molecular clone to create a recombinant virus, designated rDENV4/2 (see Fig. S1 and S2 in the supplemental material). The recombinant virus, which had 40 amino acid changes in EDIII compared to the parental wild-type (wt) DENV4 strain (Fig. 1A; see Table S1 in the supplemental material), grew to similar levels as the wt viruses in C6/36 insect cells and in a human monocytic cell line (U937) expressing dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN), a known dengue receptor, but was partially growth impaired in Vero cells (Fig. 1C). DENVs are assembled inside cells as immature virions containing premembrane proteins (prM), which are cleaved in the Golgi apparatus, resulting in the formation of mature virions. Proteolytic cleavage of prM is inefficient, and the population of virions released from infected cells is an admixture displaying different stages of maturation. As the maturation state of DENVs may influence the display of some epitopes and sensitivity to antibody neutralization (16–18), immunoblots were performed to compare the maturation state of the rDENV4/2 chimera and the wt parental strains. From C6/36 cells, DENV2 particles had high levels of prM and DENV4 particles had low levels of prM relative to E protein, indicating that DENV4 virions were more mature (Fig. 1D). The rDENV4/2 chimera had a maturation state similar to that of DENV4, indicating that insertion of EDIII from serotype 2 minimally altered the maturation state of the backbone serotype 4 virus (Fig. 1D).

Monoclonal antibody binding and neutralization of rDENV4/2. To further evaluate the impact of EDIII exchange on overall E

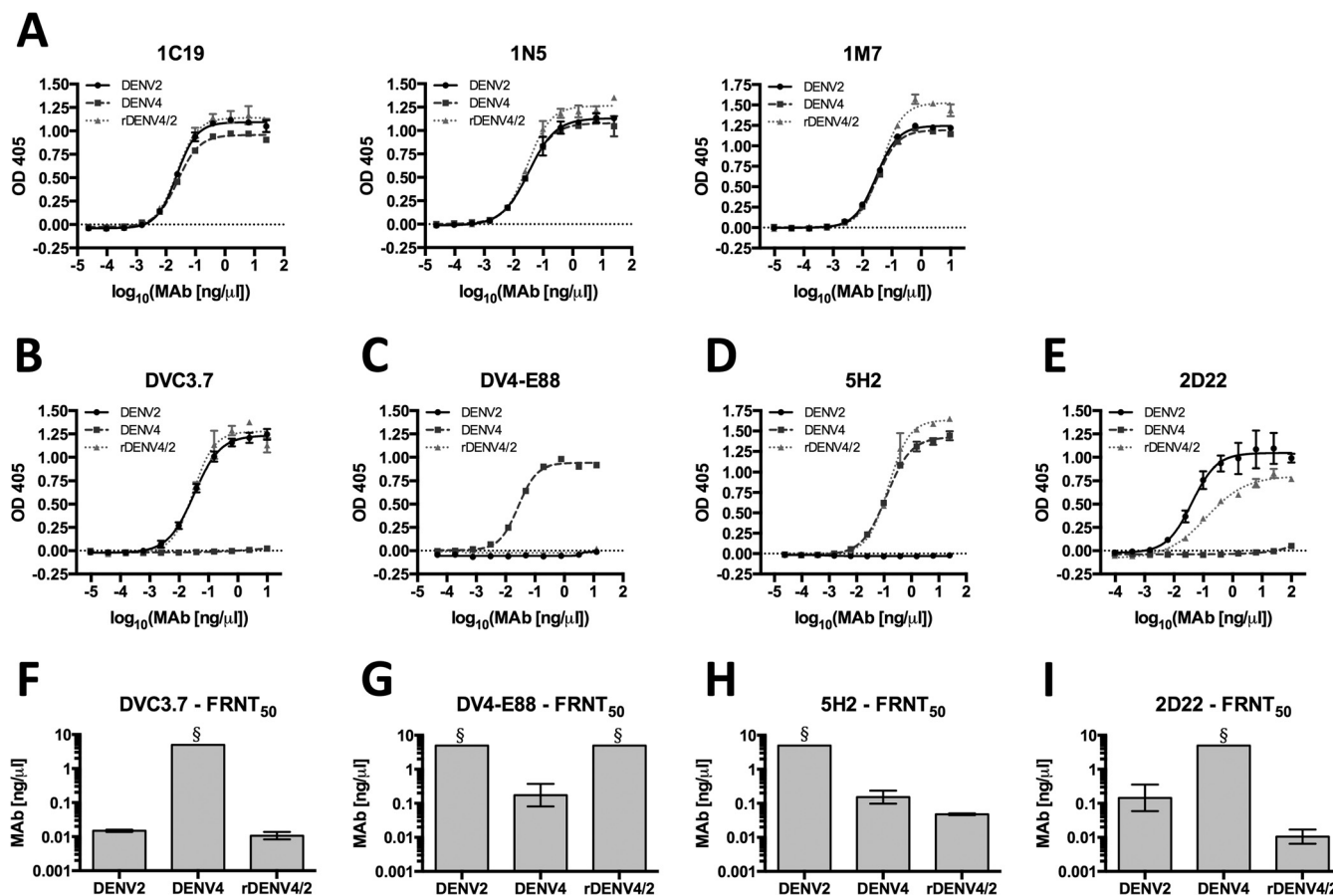


FIG 2 Recognition and neutralization of rDENV4/2 virus by DENV-specific monoclonal antibodies. Shown are the results from an ELISA capture assay with cross-reactive MABs (A), DENV2-specific EDIII MAB DVC3.7 (B), DENV4-specific EDIII MAB DV4-E88 (C), DENV4-specific EDI MAB 5H2 (D), and DENV2-specific MAB 2D22 (mean \pm SD) (E). A Vero-81 cell-based focus reduction neutralization test (FRNT) was performed using DENV2-specific EDIII MAB DVC3.7 (F), DENV4-specific EDIII MAB DV4-E88 (G), DENV4-specific EDI MAB 5H2 (H), or (I) DENV2-specific MAB 2D22 (I), and FRNT₅₀ values (i.e., the concentration of antibody required to neutralize 50% of infection) were calculated (mean \pm 95% confidence interval [CI]). §, FRNT₅₀ of >5 ng/ μ l.

protein topology and virion structure, we probed the rDENV4/2 chimera with a panel of epitope-mapped human and mouse monoclonal antibodies (see Table S2 in the supplemental material). DENV cross-reactive MABs 1C19, 1N5, and 1M7 bound to the chimera, indicating the preservation of cross-reactive epitopes (Fig. 2A) (19). DVC3.7 and DV4-E88 engage serotype-specific epitopes on EDIII of DENV2 and -4, respectively (Fig. 2B and C; see Table S2) (20, 21). Consonant with recombinant virus design, DVC3.7 bound and neutralized the chimera, whereas DV4-E88 failed to bind or neutralize the chimera (Fig. 2F and G). Binding to and neutralization by 5H2, a nonhuman primate DENV4 serotype-specific MAB with an EDI epitope, is not disrupted in rDENV4/2, showing we have not affected neutralizing epitopes present on other domains (Fig. 2D and H; see Table S2) (22). Overall, these results demonstrate that the rDENV4/2 chimera displays epitopes in a manner consistent with display on a properly folded and functional chimeric E protein. Transplantation of DENV2 EDIII into DENV4 also restored binding and neutralization by MAB 2D22, even though this antibody did not bind to DENV2 recombinant EDIII alone (Fig. 2E and I; see Table S2). We predict that the full 2D22 epitope required for antibody binding and neutralization includes EDIII, as well as some conserved residues on adjacent domains, but that the residues on EDIII alone

determine DENV2 specificity. Indeed, the cryo-electron microscopy structure of 2D22 bound to DENV2 demonstrates that the epitope consists of residues on EDIII and EDII of different monomers within a single dimer (15). As the fusion loop region of EDII is highly conserved across not only DENV but other flaviviruses as well, we predict it is the variability in EDIII that dictates 2D22 type-specificity. Of the eight contact residues identified in EDIII, only five differ between DENV2 and DENV4, suggesting these are critical residues important for 2D22 binding and neutralization (15).

Polyclonal serum neutralization of rDENV4/2. Natural primary DENV2 infections cause long-lived serotype-specific neutralizing antibody responses that can be detected for decades after exposure. To determine if “2D22-like” epitopes created by DENV2 EDIII transplantation into DENV4 were the main targets of these antibodies, neutralization assays were performed with well-characterized human and rhesus macaque dengue immune sera (see Table S3 in the supplemental material) and the rDENV4/2 and parental viruses. As expected, primary DENV2 immune sera strongly neutralized DENV2 but not DENV4 (Fig. 3A). Remarkably, in the majority of cases, these sera also efficiently neutralized the rDENV4/2 virus at levels similar to those measured with DENV2, indicating that EDIII replacement

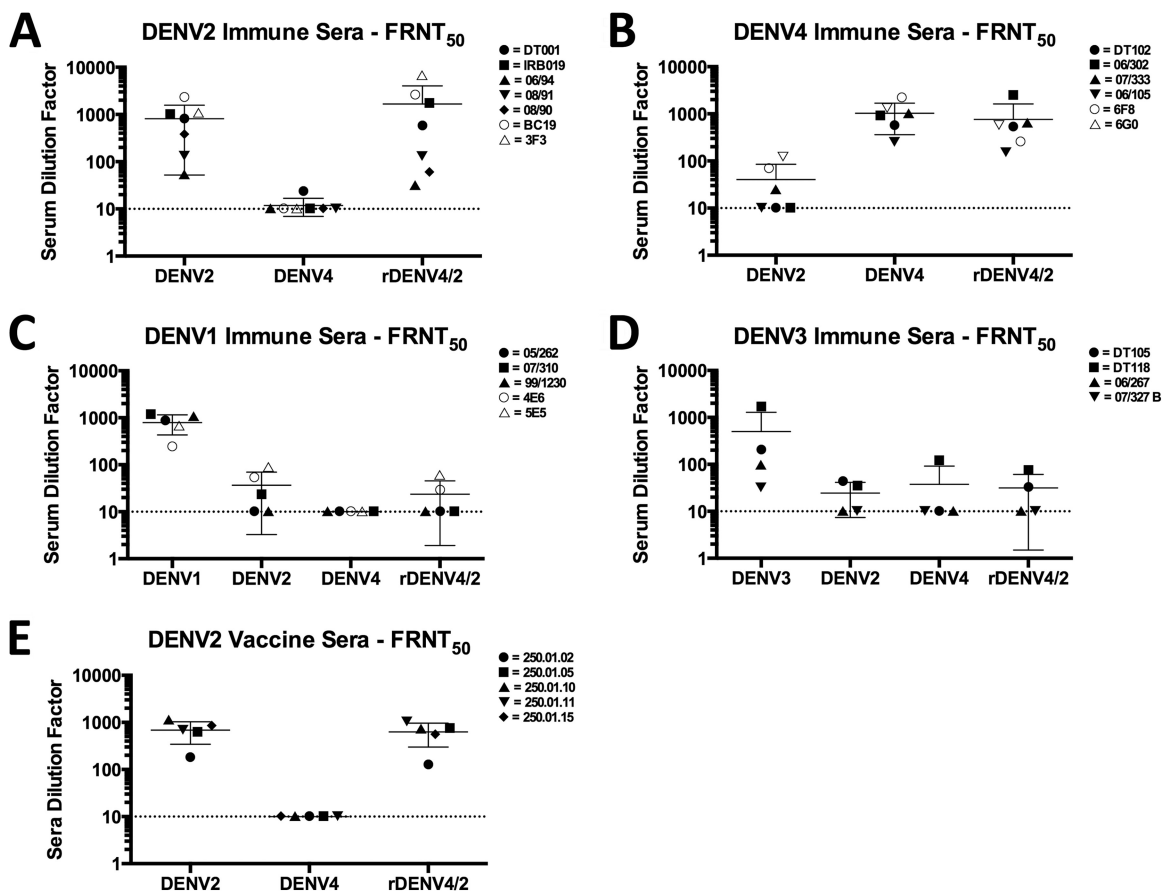


FIG 3 rDENV4/2 neutralization by human and macaque DENV immune sera. A Vero-81 cell-based focus reduction neutralization test (FRNT) was performed using primary DENV2 (A), primary DENV4 (B), primary DENV1 (C), primary DENV3 (D), and monovalent DENV2 (E) vaccine immune sera, and FRNT₅₀ values (i.e., the serum dilution factor required to neutralize 50% of infection) were calculated (mean \pm 95% CI). Solid symbols indicate human sera, and open symbols indicate rhesus macaque sera. Sera that did not block 50% of infection at the lowest serum dilution factor were assigned a value of 10 (1/2 the lower limit of detection) for graphing and statistical analysis.

was sufficient to recreate the major DENV2 neutralizing epitopes recognized by these sera. The rDENV4/2 virus remained fully sensitive to neutralization by DENV4 immune sera (Fig. 3B). These data suggest that DENV2 and DENV4 type-specific neutralizing antibodies target different epitopes on the E protein, which are both preserved on the rDENV4/2 chimera. To determine if neutralization of rDENV4/2 is specific to DENV2 and DENV4 immune sera and not reflecting a global increase in sensitivity to neutralization by any dengue immune serum, a panel of primary DENV1 and DENV3 immune sera were tested against the same three viruses. The rDENV4/2 virus did not display increased sensitivity to neutralization by DENV1 or DENV3 immune sera ($P > 0.05$ and $P > 0.05$, respectively), demonstrating that the chimeric virus was not globally sensitive to antibody neutralization (Fig. 3C and D).

DENV2 type-specific antibodies require complex epitope. To determine if the DENV2 neutralizing epitope recognized by antibodies in immune sera was entirely contained within the transplanted EDIII or included residues on EDIII and adjacent domains, human immune sera were depleted of antibodies binding intact DENV2 virions or recombinant DENV2 EDIII (Fig. 4A and B) and then tested for their ability to neutralize the rDENV4/2 virus (see Fig. S3 in the supplemental material). We have previ-

ously demonstrated that the recombinant DENV2 EDIII protein is properly folded and contains well-defined epitopes, such as the lateral ridge and A-strand epitopes recognized by some mouse and human neutralizing antibodies (23, 24). When six primary DENV2 human immune sera were depleted using DENV2 virions, between one-half to three-quarters (68% \pm 21%) of the neutralizing potency was lost, depending on the serum sample (Table 1). When the same sera were depleted using DENV2 rEDIII alone, five sera displayed minor loss in neutralization (22% \pm 3%), and one sample (DT110) lost 58% of neutralization, a significantly smaller loss of neutralization than that of DENV2 depletions (Table 1) ($P < 0.05$). These results indicate that most DENV2 epitopes targeted by polyclonal type-specific human neutralizing antibodies require assembly of more higher-order structures than simple domains or monomers of E protein. We conclude that “2D22-like” EDIII-containing quaternary epitopes are a major target of serotype 2-specific long-lived polyclonal neutralizing antibodies that develop after DENV2 infections.

To determine if dengue vaccines can induce “2D22-like” quaternary epitope-targeted neutralizing antibodies, we tested sera from 5 subjects who had developed DENV2 neutralizing antibodies after receiving a monovalent live attenuated DENV2 vaccine developed by the NIH (25). The vaccine sera neutralized DENV2

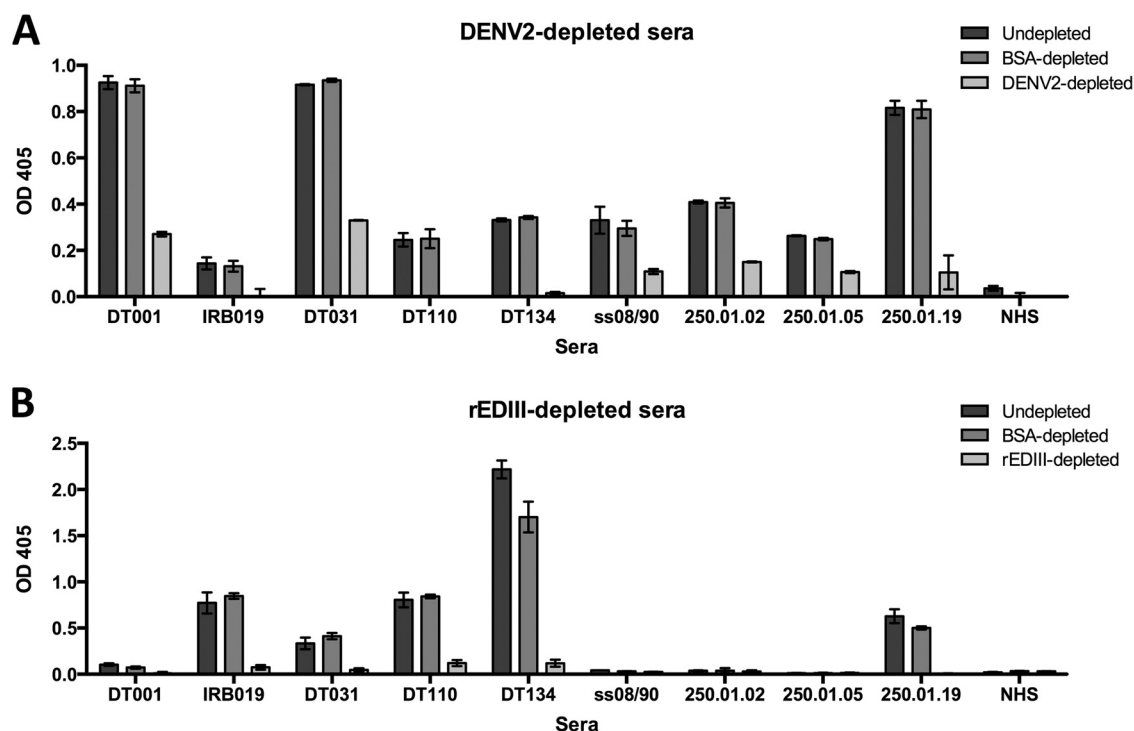


FIG 4 Depletion of DENV2- and rEDIII-binding antibodies. (A) DENV2 immune sera were depleted using polystyrene beads coated with virus (DENV2 depleted) or BSA (BSA depleted), and removal of DENV2-binding antibodies was confirmed by ELISA, with wells directly coated with DENV2 antigen. (B) DENV2 immune sera were also depleted using Dynabeads coated with DENV2 rEDIII (rEDIII depleted) or BSA (BSA depleted), and removal of DENV2 rEDIII-binding antibodies was confirmed by ELISA with wells directly coated with rEDIII protein. DT001, IRB019, D031, DT110, DT134, and ss08/90 are sera from people exposed to natural DENV2 infections. 250.01.02, 250.01.05, and 250.01.19 are sera from people who received the NIH DENV2 vaccine. NHS, normal human serum.

and the rDENV4/2 chimera but not DENV4, demonstrating that the vaccine induced neutralizing antibodies that tracked with the transplanted EDIII (Fig. 3E) ($P < 0.01$). To determine if the vaccine-induced antibodies also recognized a quaternary epitope that extended beyond EDIII, three vaccine sera were depleted of antibodies binding intact DENV2 virions or recombinant DENV2 EDIII and then tested for the ability to neutralize the chimeric virus. In all three samples, depletion with whole virus led to a nearly complete loss of neutralizing antibodies (Table 1). Removal of EDIII-specific antibodies resulted in a loss of neutralizing antibodies in one vaccine sample, while the other two samples retained the majority of neutralizing antibodies after EDIII depletion (Table 1). Thus, the vaccine induced neutralizing antibodies that bind to epitopes contained within EDIII or more complex epitopes that extend beyond EDIII.

DISCUSSION

We have described an approach using whole-domain replacement to identify principal antigenic sites targeted by polyclonal antibodies following natural DENV infection or experimental live attenuated DENV vaccination. With the rDENV4/2 chimera, we observed a clear gain of DENV2 neutralization and no loss of sensitivity to neutralization by DENV4 sera, suggesting that the principal DENV4 neutralizing epitopes are distinct from DENV2 epitopes. Importantly, these data demonstrate that a single recombinant DENV can be designed that encodes major neutralizing epitopes from two virus serotypes.

Several recent studies point to the importance of quaternary

epitopes as targets of human DENV neutralizing antibodies (10–14). DENV1 and 3 neutralizing hMAbs recognize distinct quaternary structure epitopes centered at the EDI/II hinge. However, only a small fraction (<3%) of DENV-specific memory B-cell clones produce strongly neutralizing antibodies (26). It has not been clear if epitopes defined using human MAbs are the main targets of the polyclonal serum neutralizing antibody response as well. Our studies here demonstrate that the DENV2 serotype-specific epitopes targeted by a human MAb and polyclonal immune sera are closely related if not identical. The epitope is a complex quaternary epitope and includes critical residues in EDIII that determine serotype specificity.

The results reveal the fundamental importance of complex quaternary structures on the surface of DENV particles for driving potent antibody immune responses. Our results are entirely consistent with the 2D22 epitope structure reported by Fibriansah et al. in that the antibody footprint contains critical contact residues on EDIII of one monomer, as well as the fusion loop and BC loop of EDII on the adjacent monomer, bridging across the dimer (15). The structure also demonstrates that the 2D22 antibody contact sites on EDIII are not conserved between serotypes, but the contact sites on EDII are highly conserved between DENV2 and DENV4 (15). Thus, the serotype specificity of 2D22 is determined by EDIII, and transplantation of this domain into DENV4 was sufficient to create the complete functional epitope.

Dengue vaccines have been challenging to develop because of the need to formulate vaccines with four components that simul-

TABLE 1 Neutralization of rDENV4/2 by human immune sera depleted of DENV2- or EDIII-binding antibodies^a

Serum	DENV2 virion depletion			DENV2 rEDIII depletion		
	BSA depleted (FRNT ₅₀)	DENV2 depleted (FRNT ₅₀)	% loss of neutralization (mean ± SD)	BSA depleted (FRNT ₅₀)	rEDIII depleted (FRNT ₅₀)	% loss of neutralization (mean ± SD)
DENV2 infection sera						
DT001	147	82	44	171	131	23
IRB019	931	261	72	372	305	18
DT031	1,288	721	44	1,493	1,140	24
DT110	644	76	88	1,718	729	58
DT134	235	20	91	168	129	23
ss08/90	192	64	67	468	357	24
Avg ^b			68 ± 21			28 ± 15
DENV2 vaccine sera						
250.01.02	148	<20	100	136	228	0
250.01.05	478	37	92	423	331	22
250.01.19	318	<20	100	272	<20	100
Avg			97 ± 5			41 ± 53
All sera						
Avg ^b			78 ± 22			32 ± 29

^a A Vero-81 cell-based focus reduction neutralization test (FRNT) was performed on sera depleted of DENV2- or rEDIII-binding antibodies, and FRNT₅₀ values (i.e., the serum dilution factor required to neutralize 50% of infection) were calculated as follows: % loss of neutralization = 100 − [(DENV2 or rEDIII-depleted FRNT₅₀/BSA-depleted FRNT₅₀) × 100].

^b There was a statistically significant difference between the percentage of loss of neutralization of DENV2 depleted and rEDIII depleted by two-tailed *t* test

taneously induce durable neutralizing and protective antibodies to each serotype. As the current leading dengue tetravalent vaccine resulted in increased hospitalization in children under 9 years of age, there is an urgent need for refined metrics of epitope-specific neutralization responses. Without knowing the identity of critical epitopes and regions on viruses of the four serotypes targeted by neutralizing and protective polyclonal serum antibodies, it has been difficult to dissect tetravalent vaccine responses and efficacy data from ongoing clinical trials. Epitope-exchanged noroviruses have been used to decipher complex polyclonal response patterns in vaccine samples, and it is possible the same techniques can be used with dengue tetravalent vaccine sera (27). Our results demonstrate that the NIH monovalent live attenuated DENV2 vaccine induces neutralizing antibodies that are similar to those induced by natural infection. The rDENV4/2 chimera is a powerful tool for evaluating antibody site-specific responses following infection and vaccine trials. Recombinant DENVs expressing quaternary neutralizing antibody epitopes from 2 or more serotypes may lead to simpler and more effective vaccines than current tetravalent formulations.

MATERIALS AND METHODS

Virus construction. Recombinant viruses were constructed using a four-cDNA cloning strategy, the same strategy used to create wt DENV infectious clones (see Fig. S1 in the supplemental material). Patterned after coronavirus cDNA clones (28, 29), the DENV-4 genome was subcloned into four separate cDNA plasmids. A T7 promoter was introduced into the 5′ end of the A fragment, and unique type IIS restriction endonuclease cleavage sites are introduced into the 5′ and 3′ ends of each fragment to allow for systematic assembly into a genome-length cDNA from which full-length transcripts can be derived (28–30).

The EDIII residues from DENV2 were introduced into the DENV4 A subclone by replacing E nucleotides 900 to 1179 with the corresponding nucleotides encoding variant DENV2 amino acids. The new A fragment

with nucleotides from DENV2 was synthesized and inserted into the pUC-57 plasmid (BioBasic). The new A plasmid and the DENV4 B, C, and D plasmids were grown in *Escherichia coli* cells, purified, digested with the corresponding type IIS restriction enzymes, and ligated using T4 DNA ligase to create a full-length cDNA dengue viral genome. The full-length cDNA was transcribed into genome-length RNAs using T7 polymerase, as previously described by our group (28–30). Recombinant RNA was electroporated into BHK-21 cells, and cell culture supernatant containing viable virus was harvested. Virus was then passaged two times on C6/36 cells, centrifuged to remove cellular debris, and stored at −80°C. Passage 3 represents our working stock.

Cells. Mosquito *Aedes albopictus* C6/36 cells were grown in Gibco minimal essential medium (MEM) at 32°C. Vero-81 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) and DC-SIGN-expressing U937 cells (U937+DC-SIGN cells) were maintained in RPMI at 37°C. Medium was supplemented with fetal bovine serum (FBS) (10% for Vero-81 and 5% for C6/36 and U937+DC-SIGN cells), which was lowered to 2% after infection. The C6/36 and U937+DC-SIGN media were supplemented with nonessential amino acids, and U937+DC-SIGN medium was also supplemented with L-glutamine and 2-mercaptoethanol. All media were additionally supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were incubated in 5% CO₂ as previously described by our group (30).

DENV type-specific PCR and RFLP analysis. Total RNA was isolated from viral supernatants and used as the template for cDNA synthesis using standard molecular techniques. Serotype-specific PCR and restriction fragment length polymorphism (RFLP) restriction endonuclease analyses were performed on cDNA samples in order to validate the purity of the recombinant viral preparations (see Fig. S2A, B, and C in the supplemental material).

Binding ELISA. Equal quantities of virus (as previously titrated by enzyme-linked immunosorbent assay [ELISA]) were captured using either mouse anti-DENV MABs 4G2 and 2H2 or human MAB 1C19. Primary antibodies were diluted 4-fold starting at concentrations ranging from 10 ng/μl to 100 ng/μl. Alkaline phosphatase-conjugated secondary

antibodies were used to detect binding of primary antibodies with *p*-nitrophenyl phosphate substrate, and reaction color changes were quantified by spectrophotometry, as previously described (20).

DENV immune sera. Deidentified human DENV immune sera were collected from individuals with confirmed previous natural DENV infections (see Table S2 in the supplemental material). All donations were collected in compliance with the Institutional Review Board of the University of North Carolina at Chapel Hill (protocol 08-0895). Deidentified human immune sera previously collected from adults given the NIH monovalent DENV2 vaccine (ClinicalTrials.gov identifier NCT00920517) was provided by Anna Durbin and Stephen Whitehead. All sera were collected following informed consent and approval by the Western Institutional Review Board. Nonhuman primate immune sera were collected following experimental DENV infection and kindly provided by Carlos Sariol (see Table S2) (31). All procedures were reviewed and approved by the Institute's Animal Care and Use Committee at Medical Sciences Campus, University of Puerto Rico (IACUC-UPR-MS-C) and performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) (Animal Welfare Assurance no. A3421; protocol no. 7890108, 7890208, 7890209, and 7890210).

Virus titration and FRNT. One day prior to inoculation, 24-well cell culture plates were seeded with either 5×10^4 Vero-81 cells or 1×10^5 C6/36 cells. Prior to inoculation, growth medium was removed. Virus titrations were performed by serially diluting virus stocks 10-fold and then incubating them for 1 h at 37°C. After incubation, virus dilutions were added to cells for 1 h at 37°C and then overlaid with 1 ml 1% methylcellulose in OptiMEM I (Gibco) supplemented with 2% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. After 3 to 6 days of incubation at 37°C, the overlay was removed, and cells were washed with phosphate-buffered saline (PBS) and fixed in 80% methanol. Plates were blocked with 5% instant milk made in PBS and then incubated with anti-E MAb 4G2 and anti-prM MAb 2H2, both diluted 1:500 in blocking buffer. The plates then were washed and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Sigma), diluted 1:2,500 in blocking buffer. Plates were washed, foci were developed with TrueBlue HRP substrate (KPL), and then foci were counted.

For the focus reduction neutralization test (FRNT), either MAbs or sera were diluted 4-fold and mixed with ~40 focus-forming units (FFU) of virus and then incubated for 1 h at 37°C. After incubation, virus and MAb or serum dilutions were added to cells for 1 h at 37°C, and then the overlay was added and processed as described above.

Growth curves. Either Vero or C6/36 cells were inoculated at a multiplicity of infection (MOI) of 0.01. Every 24 h, culture supernatant was harvested and centrifuged to remove cellular debris. Samples were frozen at -80°C until use. Fresh medium was replaced each day. Virus titers were determined on their propagating cell type, as described above. U937+DC-SIGN cells were infected at an initial infection of 1%, and every 12 h, a sample of cells was harvested, fixed, permeabilized, and probed with 2H2 (anti-prM antibody) conjugated to Alexa Fluor 488. Infected cells were quantified using a Guava flow cytometer (Millipore).

Immunoblotting. Virus stocks were diluted in PBS, mixed with 4× Laemmli sample buffer (Bio-Rad), and heated for 10 min at 50°C. Samples were run on 12% Protean TGX gels (Bio-Rad), transferred to polyvinylidene difluoride (PVDF) membrane, and blocked in 5% instant milk in PBS plus 0.05% Tween overnight at 4°C. Membranes were probed with 0.5 µg/ml anti-E MAb 4G2, 0.5 µg/ml anti-prM MAb 2H2, and MAb 5L20 in blocking buffer for 2 h at 37°C. After washing, HRP-conjugated anti-mouse or anti-human secondary antibodies were diluted 1:10,000 in blocking buffer and incubated for 1 h at room temperature. Membrane was exposed to chemiluminescent substrate and developed on film.

Depletion of DENV2-specific antibodies from immune sera. Polyclonal immune sera were depleted of DENV2-binding antibodies as previously described (10). Briefly, polystyrene microspheres (Polysciences catalog no. 17135) were coated with purified DENV2 antigen (Microbox

catalog no. EL-22-02-001) or the bovine serum albumin (BSA) control. Immune sera were depleted of antibodies by incubation with coated beads for 45 min at 37°C for at least three rounds, until maximum depletion of antibodies was measured. Depletions of antibodies were confirmed by ELISA.

Depletion of rEDIII-specific antibodies from immune sera. Polyclonal immune sera were depleted of rEDIII-binding antibodies as previously described for rE-binding antibodies (10). Briefly, Dynabeads (Life Technologies catalog no. 14302D) were covalently conjugated to DENV2 rEDIII protein following the manufacturer's protocol or BSA control. Immune sera were depleted of antibodies by incubation with conjugated beads for 45 min at 37°C for at least three rounds, until maximum depletion of antibodies was measured. Depletions of antibodies were confirmed by ELISA.

ELISA confirmation of DENV2 or rEDIII-depleted sera. ELISA plates were coated directly with either 50 ng of DENV2 antigen or 100 ng DENV2 rEDIII per well at 4°C overnight. Plates were blocked as described above. Undepleted, control depleted, and antigen/rEDIII-depleted sera were diluted 1:40 in blocking buffer and were incubated on plates for 1 h at 37°C. DENV2 or rEDIII reactive antibodies were detected using the secondary antibody and substrate as described above.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01461-15/-/DCSupplemental>.

Figure S1, TIF file, 2.8 MB.

Figure S2, TIF file, 2.8 MB.

Figure S3, TIF file, 2.8 MB.

Table S1, DOCX file, 0.1 MB.

Table S2, DOCX file, 0.05 MB.

Table S3, DOCX file, 0.1 MB.

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